SYNTHESIS OF GALACTOSYL CERAMIDE AND GLUCOSYL CERAMIDE BY RAT BRAIN: ASSAY PROCEDURES AND CHANGES WITH AGE

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INTRODUCTION

Galactosyl ceramide is made in mouse brain from ceramide and uridine diphospho galactose (UDPGal)[16,17]. The enzyme system, evaluated *in vitro*, shows a marked preference for 2-hydroxy fatty acids (HFA) ceramide when compared with non-hydroxy fatty acids (NFA) ceramide, although brain contains HFA and NFA cerebroside in similar amounts. We do not yet know whether this difference is due to enzyme specificity or to the existence of two different enzymes. This reaction and its unexpected specificity have been observed independently in chick brain[2] and confirmed in mouse kidney[6] and brain[10].

Glucosyl ceramide is made by a similar sugar transferase in chick brain[1] but occurs in brain only in trace amounts. It appears to be converted to gangliosides[1,22], which contain only NFA. The fatty acid composition of gangliosides differs greatly from that of galactosyl ceramide; the former contains primarily stearic acid, while the latter also contains much of the very long fatty acids. Moreover the long chain base composition of gangliosides and galactosyl ceramide also differs appreciably. Thus it appears likely that the ceramide used as substrate by the galactosyltransferase is much different than the ceramide used by the glucosyltransferase. The existence of this enzyme in mouse brain has been demonstrated[17].

Neither transferase has received much study and assay conditions have not yet been worked out. In this paper we report the development of assay procedures for the two enzymes, a comparison of their properties, and an evaluation of their activities in the brains of developing rats. Since the completion of this study a report has appeared[21] covering some of the same ground; some differences in the findings are discussed.

MATERIALS AND METHODS

Materials

Ceramides were prepared from brain cerebrosides[5] and separated into NFA

ceramide and HFA ceramide by chromatography on a tall column (116 cm × 2 cm) containing silica gel (Unisil, Clarkson Chemical Co., Williamsport, Pa.). The first eluting solvent was chloroform and the second was chloroform–methanol (99:1). Hexose-labeled [^{14}C]uridine diphospho glucose ([^{14}C]UDPGlc) and ^{14}C-UDPGal were obtained from New England Nuclear Corp. (Boston, Mass.) and diluted to a specific activity of about 2600 counts/min/nmole.

Enzyme assay procedures

Fresh or frozen rat brains were homogenized in 2.5 vol. of water, lyophilized, and stored under vacuum with P_2O_5 at —20°C. Rats about 16 days old (Sprague–Dawley strain) were generally used in developing the assay procedure since this is the age of maximal specific activity.

The dried brain was homogenized in 100 vol. of benzene with a Teflon-glass homogenizer and 0.1 ml portions were pipetted into 13 mm × 100 mm screw cap culture tubes. The tubes were previously charged with aliquots of ceramide solution in benzene, 0.2 mg in 0.2 ml. (The ceramide had to be warmed slightly just before pipetting to keep it in solution.)

To minimize the settling out of the suspended brain powder in the pipette, only 3 tubes were loaded per portion. The mixture was evaporated to dryness under a stream of nitrogen, with the tubes supported in air to maintain a low temperature. NFA ceramide was used with UDPGlc and HFA ceramide was used with UDPGal.

The tubes were immersed in ice and 0.2 ml of incubation mixture was added. This consisted of 0.1 *M* Tris (pH 7.4 at 37°C), 15 m*M* $MnCl_2$, 2 m*M* EDTA (neutralized), 1 m*M* dithiothreitol, 5 m*M* nicotinamide, and 0.16 m*M* UDPGal or UDPGlc. In the case of UDPGlc, 2 m*M* ATP (neutralized) was included. The tubes were capped, using Teflon lined caps, and shaken by hand in an ice-filled sonic cleaning tank for about 30 sec. Incubation was then carried out at 37°C in a shaker.

The radioactive cerebroside thus produced was isolated by first adding 4 ml of chloroform–methanol (2:1) containing 0.1 mg of a brain sphingolipid mixture[20], then forming an aqueous phase with 0.8 ml of 0.88% KCl[8]. The tubes were centrifuged and the lower layer was washed, first with 1 ml of methanol–water (1:1) containing 0.44% KCl, then with 1 ml of methanol–water (1:1). Insoluble material at the interface was removed by filtering the interface and lower layer through a glass pressure filter. The lipids, after evaporating the filtrate to dryness, were counted with a water–toluene–BBS-3 scintillation mixture[3]. All data reported are derived from triplicate incubations, which yielded means having a relative standard deviation of about 6% for galactosyltransferase and 9% for glucosyltransferase.

In some experiments the radioactive products were chromatographed on thin-layer chromatography (TLC) plates and characterized by radioautography as described before[16].

RESULTS

Evaluation of the work-up procedure

Radioautography of TLC plates made with the biosynthetically formed galactosyl and glucosyl ceramides revealed that nearly all of the ^{14}C was in the expected cerebroside bands. A faint band of labeled material was visible at the origin, whether or not the brain powder had been included in the incubation, showing that both sugar nucleotides contained a polar impurity or were incompletely removed by the washing procedure. In a separate incubation experiment with labeled UDPGal, the observed activities obtained by our standard work-up procedure were 5245 and 59 counts/min with and without 1 mg of brain powder from a 20-day-old rat. The comparable data with UDPGlc were 3454 and 112 counts/min.

In our previous work-up procedure[16], the lipid extract was exposed to alkaline methanolysis to remove galactolipid esters. The value of this step was investigated by working up the incubation product in the standard way, then exposing the lipid to NaOH–chloroform–methanol as before[16]. This procedure yields two washes, both of which were counted, as well as the purified cerebroside. Using UDPGal, we found 318 and 59 counts/min in the washes and 5497 counts/min in the galactosyl ceramide; the alkaline methanolysis thus removed 6% of the total lipid activity. With UDPGlc the corresponding activities were 319, 49, and 2916 counts/min; the extra treatment removed 11% of the total.

TABLE I

EFFECTS OF COFACTORS ON CEREBROSIDE SYNTHESIS BY RAT BRAIN

The values shown are percents of control activity obtained by incubating 1 mg of dry brain with 0.4 mg of ceramide (HFA or NFA) in 0.2 ml containing 0.1 *M* Tris (pH 7.4), 15 m*M* $MnCl_2$, 1 m*M* EDTA, 1 m*M* dithiothreitol, 5 m*M* nicotinamide, 2 m*M* ATP, and 0.08 m*M* [^{14}C]UDP–hexose. Incubations were for 2 h at 37°C and yielded about 1.1 nmoles of labeled cerebroside in the control tubes.

Condition		*Galactosyltransferase*	*Glucosyltransferase*
(1)	— Mn^{2+}, — EDTA	54	13
(2)	— Mn^{2+}	51	34
(3)	5 m*M* Mg^{2+}–Mn^{2+}	108	55
(4)	5 m*M* Mn^{2+}	99	62
(5)	15 m*M* Mg^{2+}–Mn^{2+}	112	75
(6)	— ATP	149	85
(7)	4 m*M* ATP	84	94
(8)	— dithiothreitol	85	72
(9)	2 m*M* dithiothreitol	98	107
(10)	— nicotinamide	90	99
(11)	10 m*M* nicotinamide	97	96
(12)	— EDTA	100	105
(13)	2 m*M* EDTA	112	110

Cofactor requirements

Examination of the effects of metal ions, ATP, dithiothreitol, nicotinamide, and EDTA showed (Table I) that both hexose transferases are stimulated by Mn^{2+} (line 2) and that some endogenous metal interferes in the synthesis of glucosyl ceramide (line 1). The galactosyltransferase can use Mg^{2+} about as well as Mn^{2+} but the other enzyme is somewhat less active with Mg^{2+} (lines 3–5).

In a more detailed study we showed that ATP inhibits the synthesis of galactosyl ceramide but, at a concentration of 2 m*M*, slightly stimulates the synthesis of glucosyl ceramide (lines 6, 7). The other components of the incubation mixture are moderately stimulatory for both enzymes (lines 8–13). While these components did not produce large stimulations individually, the effects were consistent and seemed worth utilizing.

pH requirements

There was considerable similarity in the effects of pH on both enzymes except above pH 8.2 (Fig. 1). At the higher pHs there was visible a brown color, seen also with Tris and Mn^{2+} alone. This must mean that the alkaline incubation medium is deficient in free Mn^{2+} and the glucosyltransferase, which has a greater requirement than the galactosyltransferase for Mn^{2+}, should be expected to exhibit a particularly depressed activity.

Basu *et al.*[2] found a slightly higher pH optimum (7.8 with Bicine buffer) in the formation of glucosyl ceramide by a particulate fraction from chick brain. More striking was their finding that Mn^{2+} was not stimulatory and that the optimal temperature was 32°C. With our system, the activity was lower at 32°C, measured at pH 7.4 and 7.8. Galactosyltransferase showed 56% and glucosyltransferase showed 75% of the activity at 37°C.

Relation between time of incubation and activity

The activity of both transferases remained steady for 90 min and decreased

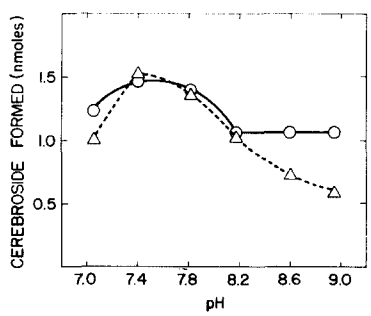

Fig. 1. Relation between pH and enzyme activity. Solid line, galactosyl ceramide formation; broken line, glucosyl ceramide. The standard assay conditions were as described in the text, but 0.2 m*M* UDP-hexose was used. Incubations were for 2 h.

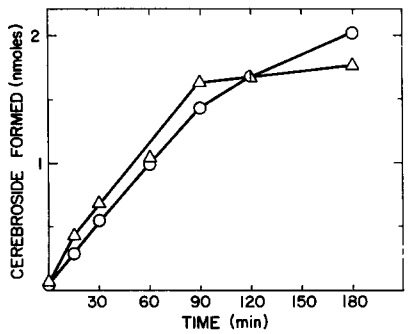

Fig. 2. Synthesis of cerebrosides as a function of time. Circles, galactosyl ceramide formation; triangles, glucosyl ceramide. Assay as in Fig. 1.

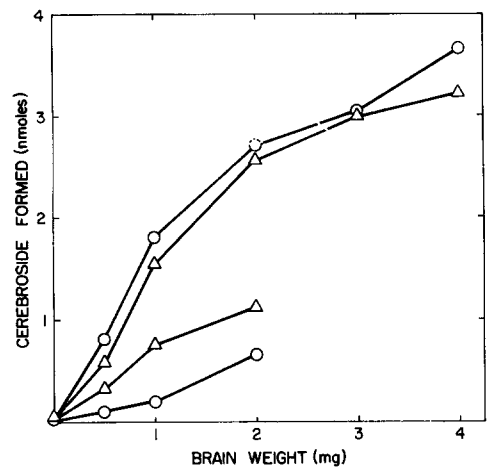

Fig. 3. Relation between enzyme weight and activity. Circles, galactosyl ceramide formation (lower curve, without added HFA ceramide). Triangles, glucosyl ceramide (lower curve, without added NFA ceramide). Assay as in Fig. 1.

afterward (Fig. 2). While the assays were routinely run for 120 min in the experiments described in this paper, better linearity would be expected with 90 min incubations.

Effect of enzyme and substrate concentration

For both enzymes the activity was proportional to weight of brain powder up to 1 mg (Fig. 3). Both curves were remarkably similar. The lower curves in Fig. 3 show that appreciable cerebroside formation can take place in the absence of added ceramide, evidently due to the availability in brain of endogenous NFA ceramide[13,19], the concentration of which is proportional to the concentration of tissue in the incubation tube.

Examination by TLC radioautography of the product obtained with labeled UDPGal in the *absence* of added ceramide showed, as with mouse microsomes[16],

that NFA galactosyl ceramide had been formed. Inclusion of HFA ceramide repressed this formation and HFA galactosyl ceramide was formed instead. The reaction with labeled UDPGlc in the absence of added ceramide similarly yielded NFA glucosyl ceramide, primarily of the long chain type (palmitate and stearate). This is consistent with the predominance of these fatty acids in brain ceramide, as shown by TLC and GLC. When NFA ceramide (containing *very long* chain fatty acids) was incubated with labeled UDPGlc, the major products were NFA glucosyl ceramides of the very long chain type and formation of the palmitoyl and stearoyl compounds was repressed. The above findings constitute proof that the exogenous ceramides were actually utilized and did not merely stimulate cerebroside formation. Similar nonspecificity with respect to chain length was noted in mouse brain microsomes[17].

When HFA ceramide was incubated with labeled UDPGlc and an acetone powder made from brain microsomes, we found that the major product was HFA glucosyl ceramide. The efficiency of utilization was similar with NFA and HFA ceramides. Evidently the glucosyltransferase is much less specific than the galactosyltransferase with respect to the presence of the hydroxyl group in the fatty acid. Shah[21] has also noted this distinction.

Varying the concentration of the added ceramide in the system described in Table I showed that 0.2 mg per incubation tube was the optimal amount for both enzymes. An increase to 0.6 mg produced a slight decrease in effectiveness (10% for galactocerebroside, 14% for glucocerebroside), possibly due to an excessively thick coating of lipid on the brain powder. Decreasing the amount of ceramide to 0.02 mg reduced the activities 20% (galactose) and 40% (glucose).

The optimal concentration of sugar nucleotide was 0.16 m*M* for both enzymes (Table II). In other experiments this concentration was found to be superior to 0.12 m*M*, but the difference was not large. At present labeled nucleotide sugars are quite expensive and difficult to make.

Variations in assay conditions

In a study of a brain galactosyltransferase acting on diglyceride, Wenger *et al.*[23] found that fresh, wet microsomes were less effective than microsomes which had

TABLE II

SYNTHESIS OF CEREBROSIDES AS A FUNCTION OF THE CONCENTRATION OF SUGAR NUCLEOTIDE

Incubation conditions as in text except that differing concentrations of UDP–hexose were used. Incubations were for 2 h.

Nucleotide concentration (μM)	*Nmoles of cerebroside formed*	
	Galactosyltransferase	*Glucosyltransferase*
40	0.81	0.68
80	1.15	1.05
160	1.89	1.57
240	1.53	1.50

TABLE III

SYNTHESIS OF CEREBROSIDES BY SOLVENT-EXTRACTED RAT BRAIN POWDER

Data show the percent change produced by the solvent treatment, compared with the activity obtained with 2 mg of lyophylized rat brain in 50 m*M* Tris (pH 7.8), 5 m*M* $MgCl_2$, 1 m*M* EDTA, 1 m*M* dithiothreitol, 5 m*M* nicotinamide, 2 m*M* ATP, and 80 μM sugar nucleotide. The lyophilized brain was extracted by homogenization at 0°C with solvent, then centrifuged and the residue dried under vacuum with P_2O_5. One portion of powder was extracted 4 times with acetone; the other portion, 2 times with acetone and 2 times with ether. Incubations lasted 2 h.

Assay condition	*Acetone-extracted*		*Acetone–ether-extracted*	
	UDPGal	*UDPGlc*	*UDPGal*	*UDPGlc*
+ 0.4 mg ceramide	—25	—39	—11	—25
— ceramide	+33	—12	+9	—3

been lyophilized and washed with acetone. The lipid substrate was added in benzene solution and the solvent was removed by evaporation. We too found a marked stimulation for galactosyl ceramide synthesis with this combination of procedures, using both microsomes and whole brain. The value of the acetone washing step was investigated with lyophilized brain and we found that acetone decreased the activity of the galactosyl- and glucosyltransferases (Table III, line 1). Curiously, in the absence of added ceramide, the activity of both enzymes appeared to be less depressed or even stimulated (line 2). Perhaps the acetone treatment did not remove the endogenous ceramide and only rendered it more accessible to the transferases. Washing with ether was less toxic than washing with additional acetone.

Our previous trials with wet mouse brain microsomes, using HFA ceramide emulsified with Tween 20, revealed that the detergent was quite inhibitory[16]. The best results were obtained by evaporating a solution of ceramide onto Celite, a purified diatomaceous earth. This procedure gave relatively poor activities when we used lyophilized brain or microsome powder, perhaps because the additional surface area introduced by the Celite withdrew part of the lipid from contact with the enzyme.

Additional attempts to use an emulsion of 0.3 or 0.4 mg of HFA ceramide were made with 2 mg of microsomal acetone powder and UDPGal. Emulsions of varying degrees of clarity were made by coevaporating solutions of emulsifier and 1 mg of HFA ceramide, then sonicating strongly in 1 ml of water. Palmitoyl dihydroxyacetone phosphate (40 μg), Triton CF54 + Cutscum (0.2 mg each), Triton X-100 (0.4 mg) + Cutscum (0.8 mg), and cerebroside sulfate + a lecithin–sphingomyelin mixture (0.15 mg each) gave almost no synthesis of cerebroside. Cerebroside sulfate (0.3 mg) as the sole emulsifying agent produced 38% of the control activity and further study of this (natural) emulsifier might be useful. Shah[21] found Tween 20 to have little effect on either cerebroside synthesizing enzyme.

Enzyme stability

The galactosyltransferase was found to be moderately stable to storage of the brain powder (see Methods section), losing about 4% activity after one day, 11%

after 7 days, and 25% after 27 days. The glucosyltransferase was somewhat more labile during the early stage, losing 16% after one day, 41% after 7 days, and 46% after 27 days.

Changes in activity as a function of age

Groups of 5 or 10 rats were sacrificed shortly after receipt of the animals, the brains were homogenized and lyophilized in groups, and assays were carried out as in the standard method. Some samples were analyzed without the addition of ceramide.

We found (Fig. 4) that the two enzymes follow rather different patterns during early life. The specific activity of the glucosyltransferase rises slightly between 4 and 16 days of age, but the galactosyltransferase increases 5-fold during a similar period (8–16 days). Subsequently both enzymes decrease in specific activity considerably, leveling off at approximately 60 days. It is notable that the 'mature' rat retains the ability to synthesize both types of cerebrosides.

Shah's data on age changes for these enzymes appeared after this work was completed[21]. His procedure differed in that microsomes (not brain) were assayed and no data were furnished on the adequacy of the medium for furnishing satisfactory assays. Moreover the type of rat used was not indicated, making a comparison even more difficult. Our data for glucosyl ceramide synthesis are rather similar but we differ somewhat in the curves for galactosyl ceramide. Shah's rats exhibited no galactosyltransferase activity at all until after 11 days and the period of rising activity extended to 30 days before dropping somewhat at 60 days. Since our study included

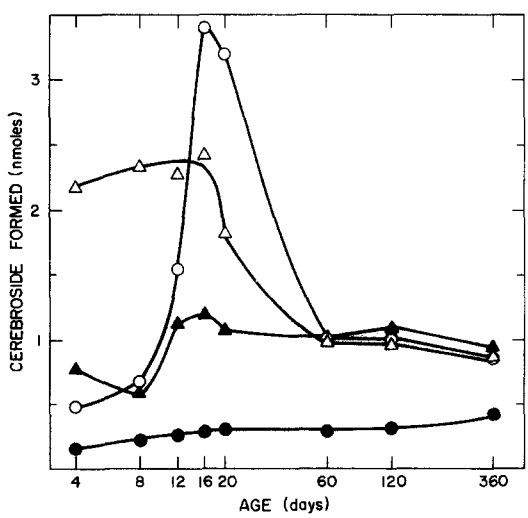

Fig. 4. Relation between rat age and sugar transferase activity. Circles, galactosyl ceramide formation (solid circles, without added ceramide). Triangles, glucosyl ceramide (solid triangles, without added ceramide). Standard assay conditions as in text, with 2 h incubations. Ten rats were used for each point under 60 days; 5 rats for the others. Note that the age scale is logarithmic so the rate of decline after 20 days is exaggerated.

only one time point (20 days) between 16 and 60 days, it seemed possible that we had missed the 20–30 day period of rising activity and we accordingly assayed a new series of rats, 20, 30, 40, and 60 days old. The specific activities for galactosyltransferase were 3.98, 2.66, 2.15, and 1.21 nmoles/mg of dry brain respectively, values which fall reasonably well on the line in Fig. 4. Only a small part of the difference between Shah's data and ours is due to the different bases of calculation. When we use protein content as the basis, our specific activities become 5.31 and 3.69 nmoles/mg of protein at 20 and 30 days, and it is evident that maximal specific activity in whole brain is reached at 16–20 days. Our activity, incidentally, is about 5 times that observed by Shah.

The specific activities for glucosyltransferase in our second series of rats were 1.33, 1.17, 1.11, and 0.83 nmoles/mg brain at 20, 30, 40, and 60 days of age. These values fall a little below the glucocerebroside curve of Fig. 4, but the trend is similar.

The activities derived from endogenous ceramide (Fig. 4) show the same difference noted in Fig. 3, namely more rapid utilization by the glucosylation enzyme. The coincidence for this enzyme between the synthetic rates with and without added ceramide after 60 days suggests that there is no functional shortage of NFA ceramide for ganglioside synthesis in later life.

Our curve for galactocerebroside synthesis matches to some extent the curve found by Hildebrand *et al.*[11] for the synthesis of psychosine in rat brain preparations, although they reported a decline to zero activity by 180 days. A similar study in mice by Neskovic *et al.*[18], covering only the period 8–70 days, yielded a curve much like ours. Another lipid galactosyltransferase, which forms galactosyl diglyceride in rat brain, also follows a time sequence much like ours[23].

Our curve for glucosyl ceramide synthesis can be compared with those of two enzymes which act later in the path of ganglioside synthesis. Hildebrand *et al.*[11] found that the galactosyltransferases which form lactosyl ceramide and ganglioside GM_1 in rat brain decline in specific activity between 1 and 180 days, little or no activity being visible at the last point. Replotting their data (based on wet weight of brain) on a dry weight basis would bring their curves closer to ours for the early time period.

DISCUSSION

Our age study adds to the picture now developing of two glycolipid systems, one involving myelin formation, the other, ganglioside formation. The enzymes needed to make myelin components, including the sulfotransferase which forms cerebroside sulfate[15], occur in brain in low concentrations until myelin formation is initiated. They reach a peak activity at about 20 days in the rat, then begin a period of decline for the next month or so. The capability of synthesizing sulfatide and galactosyl ceramide is maintained for many months longer, a point supported by chemical analysis showing marked accumulation of these glycolipids for at least 9 months[12].

In contrast, ganglioside accretion continues at a steady pace between 7 and 21 days[12]. There is then a period of slow accretion (up to approximately 52 days), and a steady level for many months. These 3 stages match our curve for glucosyl

ceramide synthesis quite well and it is possible that this enzyme is the rate-limiting factor controlling ganglioside formation.

Our finding that appreciable galactosyltransferase activity is present even at 4 days of age does not match the previous findings that galactosyl ceramide appears only after 8 days[7,9,12,14]. By the use of silica gel columns and TLC plates we were indeed able to demonstrate the presence at 4 days of minute amounts of galactosyl ceramides (NFA and HFA), as well as glucosyl ceramide. However, it would appear that some other factor, such as formation of myelin proteins, restricts the deposition of galactocerebroside prior to 8 days. It is also likely that an appreciable portion of the galactosyltransferase in the 4 and 8 day rat brain occurs in neurons, where it is relatively ineffectual in depositing galactocerebroside[4].

SUMMARY

Conditions were determined for assaying whole rat brain for the galactosyl- and glucosyltransferases which form cerebrosides from ceramide and a sugar nucleotide. The brain was homogenized in water, lyophilized, and suspended in benzene together with ceramide. The mixture of tissue and substrate was evaporated to dryness with nitrogen and then incubated in a medium containing EDTA, Tris buffer, dithiothreitol, nicotinamide, Mn^{2+}, and radioactive sugar nucleotide. In the case of UDPGlc, ATP was also added. The galactosyltransferase could use Mg^{2+} about as well as Mn^{2+} and showed a marked preference for ceramides containing a hydroxy fatty acid, while the glucosyltransferase utilized ceramides of either type equally well but was only slightly stimulated by Mg^{2+}. The pH optimum was about 7.4 for both enzymes. Other methods tested for bringing the lipoidal substrate into contact with the enzyme yielded much lower activities.

The activity of the two enzymes changed with age in different fashions during the early stage of postnatal life. Galactosyltransferase activity was low in the beginning, rising 5-fold between 8 and 16 days, while glucosyltransferase activity was high in the beginning, remaining almost constant until 16 days. Both enzymes decreased in activity over the next 40 days, reaching a plateau covering at least 300 more days.

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